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SOME DATA ON CELL INCLUSIONS AND NATURAL HOSTS OF BROAD BEAN WILT VIRUS*

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Introduction

Broad bean wilt virus (BBWV) is known as a virus having several well characterized strains, e. g. the petunia ringspot, nasturtium ringspot (NRS) etc. Some strains and isolates of BBWV produce different crystalline and amorphous inclusion bodies (Juretić et al. 1970, Sahambi et al. 1973, Miličić et al. 1974). Among various crystalline inclusions of BBWV it is necessary to point out the tubular crystals described by Rubio-Huertos (1968), which are characteristic of NRS strain (Sahambi et al. 1973). Moreover, the BBWV forms polyhedral and elongated true crystals (Rubio-Huertos 1968, Miličić et al. 1974).

Some years ago we observed that the inclusion bodies of a German NRS strain differed from inclusions of the Yugoslav NRS. While the latter strain often formed various types of crystals in infected plants, the former strain did not provoke the formation of polyhedral crystals (Juretić et al. 1970). Several other authors also found that the crystalline inclusions of various BBWV isolates differ (Sahambi 1974, Shukla and Schmelzer 1972, Taylor and Stubbs 1972).

These results have stimulated us to investigate the cell inclusions of two German and two Yugoslav isolates of BBWV. Observations performed on mentioned inclusion bodies will be presented in this paper. Besides, we shall describe here the properties of two Yugoslav isolates of BBWV found on naturally infected plants.

* Dedicated to the memory of the prominent scientist Dr. Klaus Schmelzer (Aschersleben).

Material and Methods

Two German isolates of BBWV found in *Galvezia speciosa* A. Gray and in *Celsia arcturus* (L.) Jacq. were obtained kindly from Dr. K. Schmelzer (Aschersleben). Both hosts belong to the family *Scrophulariaceae*. In these investigations two Yugoslav isolates were used too, i. e. the isolate BBWV-V found on *Vicia faba* and the isolate BBWV-D from *Digitalis ferruginea*. Both isolates were discovered in the surroundings of Zagreb.

During the investigations a serum against BBWV was employed which was sent us kindly by Dr. J. A. Tomlinson (Warwick, England).

The light microscopic investigations of cell inclusions were performed on living plant material. For electron microscope investigations strips of leaf tissue were fixed in 1 % glutaraldehyde in cacodylate buffer and after washing in buffer were postfixed in 1 % osmium tetroxide. After fixation the strips were dehydrated in ethanol and acetone and embedded in Araldite. The ultrathin sections were stained with uranyl acetate and lead citrate. and examined in a Siemens Elmiskop I.

Cytoplasmic Virus Inclusions

Very large amorphous inclusion bodies (X-bodies) were easily visible with light microscope and were usually present in epidermis cells infected with all investigated isolates of BBWV (fig. 1 A). The bodies were especially investigated on broad bean plants; they were placed in the cytoplasm, and were often larger than the nuclei. Every cell had regularly only one inclusion body which was in contact with the nucleus. Sometimes the amorphous inclusions were vacuolated (fig. 1 A).

The fine structure of amorphous bodies was also examined. The bodies were mostly built from an enormous number of membranes forming vesicles and tubules (fig. 2). A similar fine structure of amorphous

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Fig. 1. Inclusions of broad bean wilt virus in leaf epidermis cells of *Vicia faba*. A. Vacuolated amorphous inclusions (X-bodies) in cells infected with the isolate from *Galvezia*. B. Cells infected with *Digitalis* isolate. Amorphous inclusion body and polyhedral virus crystal in contact with the nucleus. C and D. Virus spindles and needles in cells infected with *Celsia* isolate. c crystal, n nucleus, x amorphous inclusion body.

Fig. 2. Fine structure of amorphous inclusion of BBWV. The zone of proliferated membranes with a large number of tubules and vesicles is visible. B lipid droplet.

Fig. 3. Ultrathin section of a needle crystal. Inset: a part of the needle under higher magnification.

Fig. 4. Symptoms caused by the isolates BBWV-D and BBWV-V of broad bean wilt virus. A and B: Infection provoked by BBWV-D isolate. A. *Chenopodium quinoa* with chlorosis on top leaves. B. *Petunia hybrida* with ringspot and mosaic. C and D: Leaves of *Vicia faba* infected by BBWV-V. C. Natural infection. D. Artificial infection.

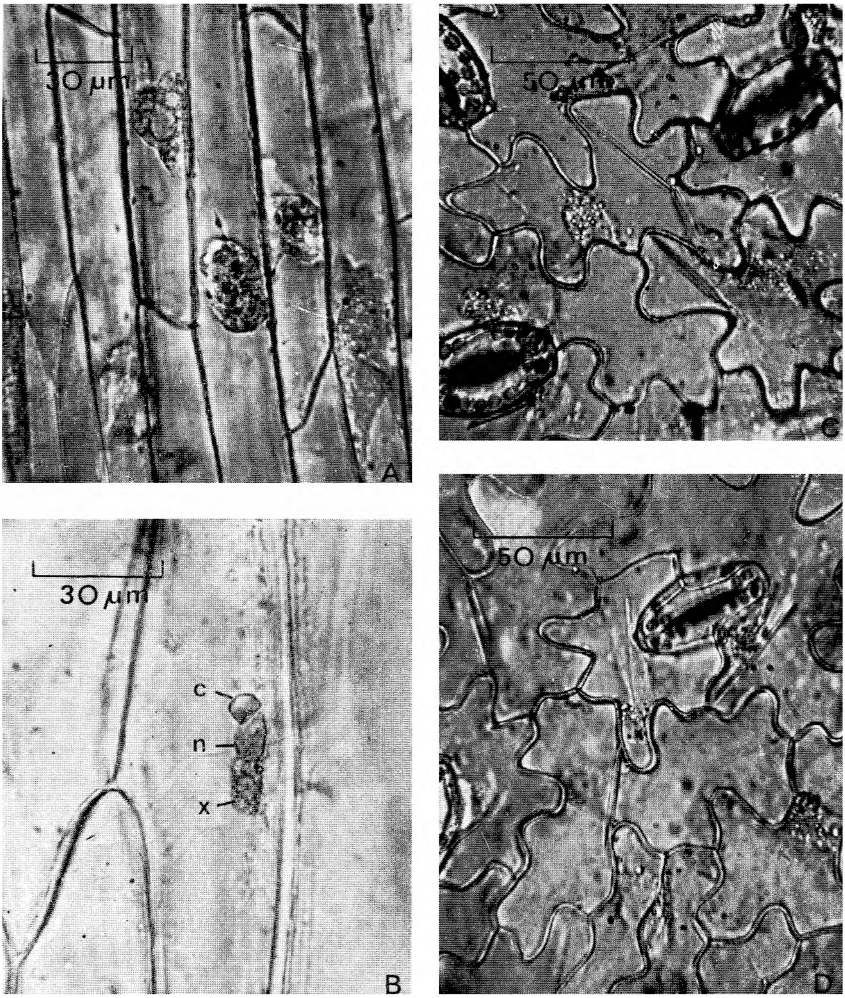
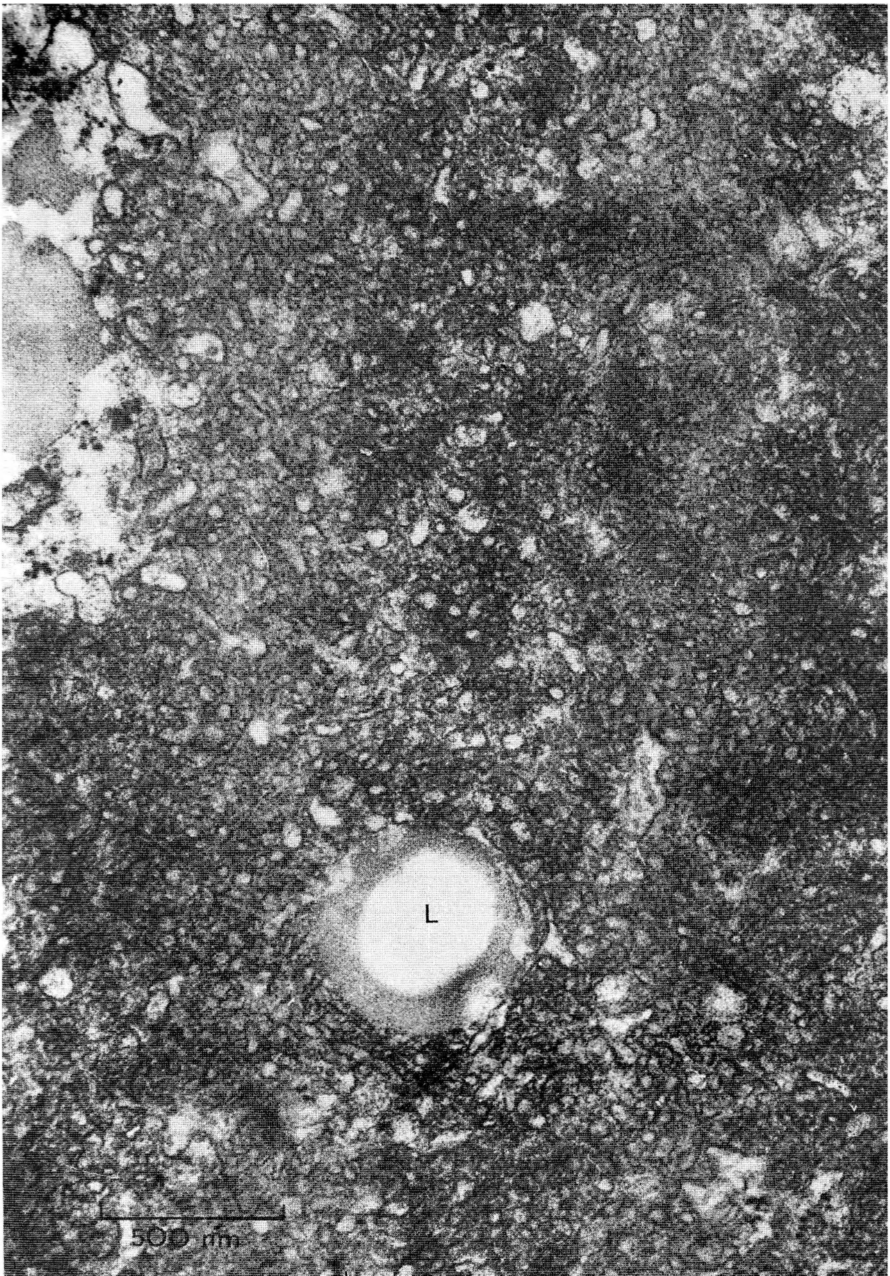


Fig. 1.



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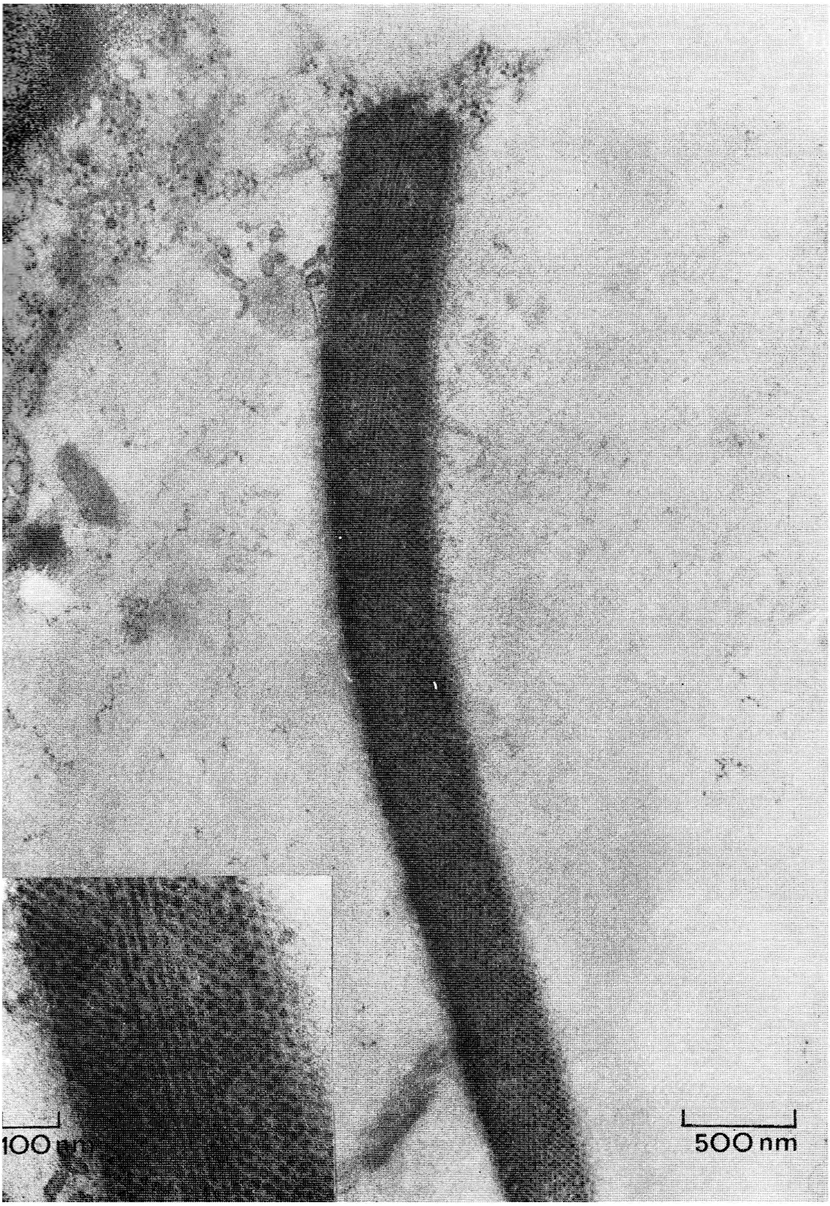

Fig. 3.

Fig. 4.

inclusions was described and illustrated by Rubio-Huertos (1968), Sahambi et. al. (1973) and Miličić et al. (1974). The membrane region was divided by Sahambi et al. in b and c zones. In zone b of this region Sahambi et al. found virus particles and they supposed that this region was involved in viral synthesis. This structure of amorphous X-bodies is an important characteristic of BBWV infection (cf. Sahambi et al. 1973, p. 163). Our observations on these structures were accomplished on inclusions of BBWV-D isolate. In these inclusions zone a, containing tubular arrays of virus particles, was not formed.

The amorphous inclusions of this isolate were often in contact with polyhedral crystals partially immersed in the amorphous mass of inclusions (fig. 1 B). Similar crystals were already described (Rubio-Huertos 1968, Juretić et al. 1970, Frowd and Tomlinson 1972), and it was found that they were built from virus particles. True BBWV crystals were observed in the vacuoles of broad bean epidermis cells infected with the *Celsia* isolate (see below), and it is very probable that they were formed primarily in the cytoplasm and later extruded into the vacuole.

Crystalline inclusions in form of needles and spindles were in places present in infected tissue and were visible even at middle magnifications of light microscope. The inclusions were found in leaves affected with isolates from *Celsia arcturus* and *Galvezia speciosa*. The spindles of these isolates frequently showed striations along their length (fig. 1 C, D). The fine structure of these spindles was not yet investigated, but it can be assumed that they are built of virus particles because of their similarity to virus spindles described by Miličić et al. (1974).

However, the needles of *Celsia* isolate were studied by electron microscope. They were built exclusively of virus particles (fig. 3), but differed with regard to structure from the elongated crystals presented by Miličić et al. (1974). The elongated crystals showed an equal regular threedimensional arrangement of virus particles in all of their parts. On the contrary, the needles of *Celsia* did not display this regularity. It is apparent from fig. 3 that the surface layer of needles has a hexagonal arrangement of particles, but in the interior layer the particles are predominantly arranged in longitudinal rows. Russo et al. (1968) observed similar conditions on crystals of tomato bushy stunt virus and explained them as a result of changes in the orientation of crystal growth.

Intravacuolar Virus Inclusions

In broad bean plants infected with the isolates from *Celsia* and *Galvezia* intravacuolar bodies were often observed even at middle magnifications of light microscope. Only one vacuolar body was ordinarily present in every cell. The body was granular and slightly smaller than the nucleus or the X-body of the same cell. Differently from X-bodies which were densely granulated, the intravacuolar bodies contained less granules, which were bound together with a transparent mucous substance. At the periphery of this vacuolar body no membrane was visible so that the body was not sharply separated from the central vacuole.

In broad bean plants infected with the *Celsia* isolate, one or more crystals were joined firmly to the vacuolar body, and they moved lively together showing characteristics of Brownian movements. These crystals had the form of octahedra. As crystals of calcium oxalate have a similar form and are often present in the central vacuole, we treated the vacuolar cristals with Lugol solution (iodine in potassium iodide). Under the influence of this solution the crystals became brown which showed that they were built of protein. Had they been built of calcium oxalate, they would have remained unstained.

As true virus crystals in form of octahedra often appear in cytoplasm or in X-bodies provoked by various isolates of BBWV (Rubio-Huertos 1972, Juretić et al. 1970), it is very probable that the mentioned intravacuolar protein crystals of *Celsia* represent virus crystals also. The octahedral crystals were sometimes observed in cytoplasm close to the X-bodies produced under the influence of the *Celsia* isolate. It is very probable that the crystals together with the parts of altered cytoplasm were extruded in the central vacuole where they assumed the form of the described intravacuolar bodies.

That these inclusions were placed in the central vacuole it was concluded in the first place on account of their Brownian movements. This opinion was supported by the fact that they were always placed in the middle of the cell where the central vacuole is situated, but never in the parietal cytoplasm near the cell wall.

New Natural Hosts of Broad Bean Wilt Virus in Yugoslavia

BBWV has been known only in *Tropaeolum majus* in Yugoslavia till now. However, during this investigation the isolate BBWV-D was obtained from *Digitalis ferruginea* cultivated in the Botanical Garden in Zagreb. *D. ferruginea* is spread as a wild plant in all parts of Yugoslavia. According to the data of Schumann (1963) this species can be affected by a complex disease, *Digitalis* mosaic, which is caused by ribgrass mosaic virus and/or NRS virus. On the other hand, the specimens of *D. ferruginea* from Zagreb were infected only with BBWV. These plants displayed symptoms in form of leaf mottling. This virus provoked only one form of crystalline inclusions, i. e. the polyhedral crystals (fig. 1 B). Due to this property it differed from the BBWV strain which was spread on *Tropaeolum majus* in the surroundigs of Zagreb. This latter strain produced three forms of crystalline inclusions, i. e. the polyhedral crystals, the elongated crystals and the virus spindles (Juretić et al. 1970).

The second new natural host of BBWV in Yugoslavia was broad bean (*Vicia faba*). Broad bean plants infected with BBWV-V were cultivated in fields in the environs of Zagreb (fig. 4 C). This host contained all three forms of crystalline inclusion bodies, and due to this property BBWV-V was equal to the normal form of BBWV spread to *Tropaeolum majus* in the region of Zagreb.

Both Yugoslav isolates were analysed on several herbaceous plants. The result of the analysis are presented in table 1.

Symptoms of both isolates on herbaceous plants were very similar, but the symptoms displayed by BBWV-V were more intense.

Afterwards the Yugoslav isolates were investigated with the serum against BBWV. Both isolates gave a positive serological reaction in double-diffusion agar gel tests.

T a b l e 1. Symptoms of isolates BBWV-D and BBWV-V on herbaceous plants

Host plant	Symptoms in inoculated leaves	Symptoms in systemically infected leaves
Chenopodium quinoa Willd.	Chlorotic local lesions	Chlorosis with epinasty and necrotic zones on top leaves (fig. 4 A)
Vicia faba L.	—	Dark green and yellow mottle, deformations, infected plant stunted (fig. 4 D)
Tetragonia expansa Thunb.	Chlorotic local lesions with diffuse margins	Mottling, deformations
Nicotiana glutinosa L.	Occasional chlorotic rings	Mottling, chlorotic ringspot and lines
Nicotiana megalosiphon Heurck et Muell. Arg	Chlorotic and necrotic lesions	Variegation with necrotic lines
Petunia hybrida hort. ex. Vilm	—	Mosaic, chlorotic ringspot (fig. 4 B)

Discussion

In this paper the properties, especially the intracellular inclusion bodies, of four isolates of BBWV were described. Two isolates originated from Yugoslavia and two from the German Democratic Republic. The Yugoslav isolate, found on *Vicia faba*, corresponded completely according to the form of inclusion bodies and other properties to the NRS strain which is very spread on *Tropaeolum majus* in the environs of Zagreb, and which was described by J u r e t i ć et al. (1970) and M i l i č i ć et al. (1974). It is especially characteristic of this strain that it causes three types of crystalline inclusions (cf. p. 20).

The second Yugoslav isolate (BBWV-D) differed from the mentioned Yugoslav *Tropaeolum* strain because it caused the formation only of one type of virus crystals, i. e. of polyhedral crystals.

With regard to the German BBWV isolates, the isolate from *Galvezia speciosa,* which derived from the Botanical Garden in Halle, was thoroughly investigated. According to the serological properties this isolate belongs to the common form of BBWV which is spread in German Democratic Republic (S c h m e l z e r 1974). The knowledge of crystalline inclusions of German isolates is still scant. It seems that the polyhedral crystals do not appear in plants infected with the German isolate from *Tropaeolum* (J u r e t i ć et al. 1970, p. 23). S h u k l a and S c h m e l z e r (1972, p. 155) were unable to find any crystalline virus inclusions in plants infected with a German isolate of BBWV from *Sinapis.* We had the possibility to establish with certainty that the polyhedral crystals are occasionally present in the vacuoles of plants infected with the isolate from *Celsia arcturus.* The natural habitat of this *Celsia* is Asia Minor and the island of Crete, but the infected plant was collected in GDR.

The occurrence of protein crystals in the vacuoles of infected *Celsia arcturus* is worth mentioning. It has been known for a long time that after virus infections various changes can appear in plant vacuoles. It seems that Miličić and Plavšić (1956) were first to transmit the cytoplasmic inclusions, i. e. virus spindles, together with intravacuolar inclusions in form of druses by means of inoculation tests from diseased plants to healthy specimens. Afterwards Kenda (1961) and Miličić (1963) confirmed these experiments, but it remained unknown whether the vacuolar bodies were metabolic products or virus particles.

Recently Russo et al. (1968), Russo and Martelli (1973), Vovlas et al. (1973), Šarić and Wrischer (1975) and other authors found that virus particles can be extruded into the vacuole where virus aggregates sometimes appear in form of crystalline inclusions.

During the investigations BBWV was found in two new host plants for Yugoslavia, i. e. in *Vicia faba* and *Digitalis ferruginea*. The last plant is a new natural host of this virus (cf. Shukla and Schmelzer 1972). On the basis of these data it can be concluded that the BBWV is more spread in Yugoslavia than it was earlier assumed.

Summary

Virus inclusion bodies of four isolates of broad bean wilt virus (BBWV) were investigated with light and electron microscope. Two virus isolates derived from Yugoslavia and two isolates from the German Democratic Republic. All these isolates ordinarily formed a large number of amorphous inclusions (X-bodies) the fine structure of which is characteristic of BBWV infections.

The first Yugoslav isolate from *Vicia faba* provoked three forms of crystalline inclusions. Because of this and other properties, it was obvious that it belonged to the nasturtium strain which is wide spread in this country. The second Yugoslav isolate from *Digitalis ferruginea* contained only one form of crystalline bodies, i. e. the polyhedral crystals. This species is a new natural host of BBWV.

The first German isolate from *Celsia arcturus* is very similar to the strain of BBWV which is common in Germany. It produced spindle- and needle-like crystals in the cytoplasm of infected cells and polyhedral crystals in the vacuole. The fine structure of needle-like virus crystals was described; it differed somewhat from the structure of elongated crystals. The second German isolate from *Galvezia speciosa* corresponded according to the inclusions in infected cells to the first German isolate, except for the polyhedral crystals which were not present.

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SADRŽAJ

NEKOLIKO PODATAKA O STANIČNIM INKLUZIJAMA I PRIRODNIM DOMADARIMA VIRUSA VENUĆA BOBA (BROAD BEAN WILT VIRUS)

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Virus venuća boba (VVB) malen je izodijametrični virus s promjerom oko 30 nm. Njegove se populacije sastoje od tri vrste čestica koje u svojoj unutrašnjosti imaju četiri vrste RNK. Broj prirodnih domadara toga virusa nije vrlo velik. Poznato je da VVB napada neke kultivirane biljke, kao npr. vrste *Vicia faba, Nicotiana tabacum, Tropaeolum majus, Catalpa bignonioides* i dr.

VVB su prvi put pronašli u Jugoslaviji na dragoljubu Juretić i sur. (1970). Pri tom su ustanovili da VVB u inficiranim stanicama svojih domadara stvara tri oblika kristaličnih inkluzija, i to poliedrične kristale, produžene kristale i proteinska vretena. Dosad je u više navrata nađen VVB na dragoljubu na području Zagreba i stvarao je jednake inkluzije.

Budući da drugi izolati VVB ne stvaraju te tri određene vrste kristala, smatramo da su one karakteristične za soj VVB koji je prilično raširen u okolici Zagreba. Nalaz VVB u okolici Zagreba na vrsti *Vicia faba*, u čijim su stanicama bile nazočne također sve te tri forme kristala, potvrdio je naše mišljenje. Tijekom ovih istraživanja pronašli smo VVB i na vrsti *Digitalis ferruginea* koja se uzgajala u Botaničkom vrstu u Zagrebu. Za razliku od navedenih izolata iz dragoljuba i boba, izolat iz *D. ferruginea* stvarao je samo poliedrične kristale. Prema tome, čini se, u okolici Zagreba raširene su dvije vrste izolata VVB od kojih jedna stvara tri oblika kristaličnih virusnih inkluzija a druga samo jedan oblik.

Prilikom istraživanja virusnih inkluzija u stanicama boba inficiranog s izolatom iz vrste *D. ferruginea* ustanovili smo da su X-tijela izgrađena od velikog broja vezikula i tubula koji tvore područja bogata membranama. Ta membranska područja karakteristična su za infekciju s VVB.

Osim toga istražili smo radi usporedbe dva njemačka izolata VVB. Prvi izolat iz vrste *Celsia arcturus* bio je po svojstvima vrlo sličan običnom tipu VVB koji je raširen u Demokratskoj Republici Njemačkoj. On je stvarao vretenaste i igličaste kristale u citoplazmi te poliedrične kristale u vakuoli. Opisana je točno fina struktura igličastih kristala. Drugi njemački izolat potjecao je iz vrste *Galvezia speciosa*. S obzirom na inkluzije taj izolat bio je sličan izolatu iz vrste *C. arcturus*. U stanicama boba inficiranim s drugim njemačkim izolatom nismo mogli pronaći poliedričnih kristala.

Iz ovog prikaza izlazi da bi dobro poznavanje inkluzija VVB moglo omogućiti brzu identifikaciju virusa i služiti čak za razlikovanje njegovih sojeva.

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